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Persistence of Avian Influenza Virus (H5N1) in Feathers Detached from Bodies of Infected Domestic Ducks▽

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Asian lineage highly pathogenic avian influenza virus (H5N1) continues to cause mortality in poultry and wild bird populations at a panzootic scale. However, little is known about its persistence in contaminated tissues derived from infected birds. We investigated avian influenza virus (H5N1) persistence in feathers detached from bodies of infected ducks to evaluate their potential risk for environmental contamination. Four-week-old domestic ducks were inoculated with different clades of avian influenza virus (H5N1). Feathers, drinking water, and feces were collected on day 3 postinoculation and stored at 4°C or 20°C. Viral persistence in samples was investigated for 360 days by virus isolation and reverse transcription-PCR. Infectious viruses persisted for the longest period in feathers, compared with drinking water and feces, at both 4°C and 20°C. Viral infectivity persisted in the feathers for 160 days at 4°C and for 15 days at 20°C. Viral titers of $10^{4.3}$ 50% egg infectious doses/ml or greater were detected for 120 days in feathers stored at 4°C. Viral RNA in feathers was more stable than the infectivity. These results indicate that feathers detached from domestic ducks infected with highly pathogenic avian influenza virus (H5N1) can be a source of environmental contamination and may function as fomites with high viral loads in the environment.

Since 1997, Asian lineage highly pathogenic avian influenza (AI) virus (H5N1) has spread from Asia to Europe, the Middle East, and Africa, causing profound economic losses in the poultry industry (3, 19). The virus has also been associated with significant mortality in wild birds (7, 20, 21, 43). Furthermore, humans and some other species of mammals have contracted the disease by close contact with infected birds (2, 10, 13, 18, 34).

AI viruses are generally perpetuated as low-pathogenicity viruses among wild aquatic birds (26, 48). This virus is mainly transmitted in waterfowl by indirect fecal-oral route through contaminated open water in fields (14, 23). Some low-pathogenicity AI outbreaks in poultry have been potentially associated with nearby waterfowl habitats (12, 17, 31). Therefore, excretion of AI virus by infected birds into the environment is epidemiologically important when attempting to control the disease.

Many factors related to the virus and environment can influence viral persistence outside the infected host (32, 37, 46). Some reports investigated the persistence of influenza viruses in environmental media such as bird feces and river water as well as on various environmental surfaces (1, 6, 11, 22, 30, 41, 42, 47, 51). However, despite the profound mortality caused by highly pathogenic AI virus (H5N1) in a number of chickens and wild birds, its persistence in virus-contaminated organs or tissues, such as carcasses of infected birds, remains to be elucidated (37). A few studies have documented the effectiveness of thermal inactivation and composting of carcasses from a safety perspective (16, 29, 39, 40). Tissue derived from infected birds can become a source of environmental contamination (37). In addition, contaminated tissue can be a source of direct infection for other animals or humans because it can become a target for scavenging wildlife (50) or food for domestic animals and humans (2, 18, 34) or because its disposal requires human handling (44).

A major cause of the spread of Asian lineage highly pathogenic AI virus (H5N1) remains unclear (8, 25, 27, 49). However, some epidemiological studies revealed that free-range domestic ducks played a prominent role in regional spread of AI (H5N1) virus in Southeast Asia (9, 24, 33). Asymptomatically infected domestic ducks can shed the virus continuously from their oral cavity and cloaca, contributing significantly to silent spread of AI virus (H5N1) (15, 33, 38). We previously reported that AI virus (H5N1) can replicate in feather epidermal cells in ducks, geese, and swans (53, 55). Feathers easily drop off the body, and infected feathers have the potential to cause environmental contamination. In addition, waterfowl feathers used for commercial purpose (45) can spread the virus to distant areas if unprocessed feathers are contaminated with the virus.

In the present study, we investigated the persistence of AI virus (H5N1) in feathers of infected domestic ducks to evaluate the potential risk for viral transmission. In particular, we hypothesized that quantitative, long-term evaluation of viral persistence in feathers detached from the body would provide insight into risk analyses involving viral persistence in contaminated tissues in the field.

MATERIALS AND METHODS

Animals. Domestic ducks (*Anas platyrhyncha* var. *domestica*) were obtained from a breeder at 1 day of age and were raised on commercial food in an isolated facility (53). All experimental procedures that involved the birds were approved by the Ethics Committee of the National Institute of Animal Health, Japan (authorization number 07-118).

Virus. We used two highly pathogenic AI viruses (H5N1), A/chicken/Miyazaki/K11/2007 (Ck/Miya/K11/07) and A/whooper swan/Akita/1/2008 (Ws/Akita/1/08).

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TABLE 1. Results of virus isolation and RT-PCR in stored samples derived from domestic ducks inoculated with AI viruses (H5N1)[a]

Day[b]	Viral titer (RT-PCR result)[c]											
	Ck/Miya/K11/07						Ws/Akita/1/08					
	4°C			20°C			4°C			20°C		
	Feathers	Water	Feces	Feathers	Water	Feces	Feathers	Water	Feces	Feathers	Water	Feces
0				6.5 (+)	1.8 (+)	− (−)				4.5 (+)	2.3 (+)	− (−)
3	5.3 (+)	2.3 (+)	− (−)	4.0 (+)	− (+)	− (−)	5.5 (+)	2.3 (−)	− (−)	5.8 (+)	− (+)	− (+)
6	5.5 (+)	2.3 (+)	2.5 (−)	4.5 (+)	− (+)	− (−)	6.8 (+)	1.8 (+)	− (−)	6.5 (+)	− (+)	− (−)
10	5.5 (+)	− (+)	− (−)	2.3 (+)	− (−)	− (−)	6.3 (+)	− (−)	− (−)	5.3 (+)	− (−)	− (−)
15	4.3 (+)	1.8 (+)	− (−)	− (+)	− (−)	− (−)	6.5 (+)	− (−)	− (−)	3.0 (+)	− (−)	− (−)
20	5.8 (+)	− (−)	− (−)	− (+)	− (−)	− (−)	7.4 (+)	− (−)	− (−)	− (+)	− (−)	− (−)
30	5.5 (+)	1.8 (+)	− (−)	− (−)	− (−)	− (−)	6.8 (+)	− (+)	− (−)	− (+)	− (−)	− (−)
40	4.5 (+)	− (−)	− (−)	− (−)	− (−)	− (−)	5.8 (+)	− (+)	− (−)	− (+)	− (−)	− (−)
50	4.8 (+)	− (−)	− (−)	− (−)	− (−)	− (−)	6.8 (+)	− (−)	− (−)	− (−)	− (−)	− (−)
60	5.3 (+)	− (−)		− (−)	− (−)		5.5 (+)	− (−)		− (−)	− (−)	
70	4.8 (+)	− (−)		− (−)	− (−)		5.8 (+)	− (−)		− (−)	− (−)	
80	4.5 (+)	− (−)		− (−)	− (−)		6.5 (+)	− (−)		− (−)	− (−)	
100	4.3 (+)	− (−)		− (−)	− (−)		4.8 (+)	− (−)		− (−)	− (−)	
120	5.3 (+)						7.5 (+)					
160	3.3 (+)						3.3 (+)					
200	− (+)						− (+)					
240	− (+)						− (+)					
280	− (+)						− (+)					
320	− (+)						− (+)					
360	− (+)						− (+)					

[a] Investigation was completed when each sample produced repeated negative results in virus isolation and RT-PCR.
[b] Day 0 means a sampling day from domestic ducks 3 days after inoculation. The initial titers were determined on a sampling day at room temperature and described in cells of 20°C.
[c] Viral titer is expressed as EID_{50}/ml; −, negative for virus isolation ($<10^{1.6}$ EID_{50}/ml). RT-PCR result: +, positive; −, negative.

Ck/Miya/K11/07 belongs to clade 2.2, which has spread over the Eastern Hemisphere (3, 43). Ws/Akita/1/08 belongs to clade 2.3.2 (43). The stock virus was propagated for 2 days in the allantoic cavity of 10-day-old embryonated chicken eggs at 37°C. Fresh, infectious allantoic fluid was harvested and stored at −80°C until use.

Viral persistence in feathers plucked from infected domestic ducks. We established two inoculation groups for viruses Ck/Miya/K11/07 and Ws/Akita/1/08. Each group was kept in a single negative-pressure isolator at biosafety level 3-approved laboratories during experimental infection. Commercial mineral water (pH 6.7; sodium, 6.5 mg/liter; hardness, 30 mg/liter) was used as drinking water, because the chlorine content in tap water could inactivate the virus (28) and also affect viral persistence in drinking water. Food and drinking water (500 ml) were replenished twice a day. Preinoculation sera from birds were assessed by hemagglutination inhibition tests and were negative for antibodies against the viruses.

Four-week-old domestic ducks ($n = 5$) in each group were inoculated intranasally with 10^7 50% egg infectious doses (EID_{50}) of each virus. The birds were monitored and euthanized 3 days after inoculation, as active viral replication was observed in the feathers at this time point in our previous study performed under the same experimental conditions (54). Feathers, drinking water, and fecal samples from each group were collected separately in 50-ml polypropylene centrifuge tubes or 125-ml polystyrene storage bottles. At least 100 contour feathers were plucked from the carcass of each bird. Only the feather calamus, which is the basal part of the feather shaft (54), was cut and collected. These were then pooled into one sample for each group. A total of 40 ml drinking water was sampled, and the supernatant was recovered after centrifugation at $3,000 \times g$ for 15 min; 5 g fresh feces was collected from the isolator pan and from the intestines of the birds after necropsy. Titration was performed to determine the initial amount of virus present, and viral RNA was determined with reverse transcription-PCR (RT-PCR) on the day of sampling (day 0). Each sample was then divided into two parts and placed in incubators set at 4°C or 20°C. Isolation of the infectious virus and viral RNA detection were performed at different time periods from day 3 through day 360. The investigation was deemed complete when each sample produced repeated negative results in virus isolation and viral RNA detection.

Virus isolation. Virus titers in the feathers, drinking water, and feces were determined using 10- or 11-day-old embryonated chicken eggs and are expressed as EID_{50}/ml. For feathers, the supernatant of 10% (wt/vol) homogenate of 4 to 5 feather calami was titrated starting with an initial 10-fold dilution in phosphate-buffered saline supplemented with antibiotics. Recovery of the infectious virus was determined by the positivity of allantoic fluids for hemagglutination activity or by the commercial antigen detection kit QuickVue Influenza A+B (Quidel Corp., San Diego, CA). Blind passage of allantoic fluids was performed when the chicken embryo was confirmed dead with negative results for virus recovery. A virus titer of $<10^{1.6}$ EID_{50}/ml was considered negative for virus isolation.

RT-PCR. One-step RT-PCR (SuperScript III one-step RT-PCR system; Invitrogen, Carlsbad, CA) was performed on total RNA extracted from the same samples used for virus isolation to detect AI virus (H5N1) gene. The primers used were H5–248–270F and H5–671–647R (52). The PCR product was electrophoresed, and viral RNA detection was confirmed by the expected band size of 424 bp.

RESULTS

Clinical signs observed were corneal opacity in two Ck/Miya/K11/07-inoculated ducks and in one Ws/Akita/1/08-inoculated duck. Other ducks were clinically healthy until their euthanization 3 days after inoculation. The initial titers for the pooled feather calami were $10^{6.5}$ EID_{50}/ml for Ck/Miya/K11/07 and $10^{4.5}$ EID_{50}/ml for Ws/Akita/1/08 (Table 1).

Infectious virus was isolated up to day 160 from feathers stored at 4°C (Table 1). Viral titers of $10^{4.3}$ EID_{50}/ml or greater were detected for 120 days in feathers stored at 4°C. In feathers stored at 20°C, viruses were isolated for a maximum period of 15 days. Although the feather samples showed some variability in viral titer over time, this variability is believed to have resulted from using pooled feathers from a number of infected birds with unequal viral distribution among feathers. RT-PCR using the same samples as for viral isolation revealed that viral RNA in feathers was more stable than viral infectivity (Table

1). Viral RNA was detected in feathers at 4°C up to day 360 (the time of study completion).

Initial viral titers in drinking water were $10^{1.8}$ EID_{50}/ml for Ck/Miya/K11/07 and $10^{2.3}$ EID_{50}/ml for Ws/Akita/1/08. Low viral titers not exceeding $10^{2.3}$ EID_{50}/ml were inconsistently detected in drinking water at 4°C over a maximum period of 30 days. No virus was isolated from drinking water at 20°C from day 3 or from feces on the sampling day (day 0). AI virus (H5N1) was isolated only from a fecal sample at 4°C on day 6 with a titer of $10^{2.5}$ EID_{50}/ml. The inconsistent results reported for drinking water and feces over the first 30 days postsampling are believed to be due to the unequal distribution of a small amount of virus in the samples.

DISCUSSION

We previously reported replication of AI virus (H5N1) in feather epidermal cells of domestic ducks, thus demonstrating the possibility of viral release from feathers (53, 54). The results of our present study further emphasize the possible role of feathers in environmental contamination. When feathers detach from the body during the active phase of viral replication, the infectious virus can be recovered from the stored feather tissue for a time period dependent on the storage temperature. The most interesting finding is that $10^{4.3}$ EID_{50}/ml or more of infectious virus persisted for at least 120 days in feather tissue stored at 4°C. Viral infectivity persisted up to 15 days in feathers stored at 20°C, and the virus could be detected for the longest duration in feathers stored at both 4°C and 20°C compared to feces from infected birds or water contaminated during the experimental infection. Whereas contaminated water or feces can be quickly diluted in the environment, contaminated tissues such as feathers with high viral loads can exist as solid materials in the field. Direct environmental contamination from these infected feathers may be limited to a local area because of the nature of solid materials but could also occur in waterfowl habitats where AI virus (H5N1) exists. Similarly, other contaminated tissues such as the carcass pose a possible risk for environmental contamination.

Our data suggest that infected feathers detached from the body for whatever reason could contaminate the environment. The following are some possible situations: mass culling of infected ducks after outbreaks of AI virus (H5N1), defeathering of domestic ducks with asymptomatic infection at slaughter, and trading of unprocessed waterfowl feathers leading to virus spread via contaminated feathers. Furthermore, although clinical symptoms such as feather loss were not observed in domestic ducks in the experimental infection, molting can be induced in birds by stress or critical disease conditions in the field.

From another perspective, the epidemiological importance of contaminated feathers may be their potential as a source of direct infection for other animals or humans. AI virus (H5N1) infection in humans in the Republic of Azerbaijan is suspected to have been caused by defeathering of infected wild swans (10). We previously reported that AI virus (H5N1) can replicate in wild swan feathers, demonstrating the zoonotic potential of feathers as a source of AI virus (H5N1) infection (55). In addition to waterfowl, histological evidence of the presence of AI virus (H5N1) in feathers has been reported for chickens experimentally infected with the virus (56).

The lack of a standard protocol for analyzing the persistence of influenza virus in the environment makes it difficult to compare results from related studies. Several factors such as temperature, relative humidity, salinity, and pH can influence the persistence of AI virus in a variety of media such as distilled water, allantoic fluids, and river water (4, 5, 35, 36, 46, 47, 51). Similar to the studies above, a low temperature of 4°C effectively increased viral persistence in feathers and drinking water. Although the actual effect of relative humidity was not determined in the present study, all samples are suspected to have been maintained in relatively high humidity, as they were completely sealed in a tube or bottle. As has been observed with water and fecal samples in the present study, the initial amount of virus in the samples can also affect the experimental result in terms of viral persistence.

One limitation of our study is that the data were collected under experimental conditions that may not be applicable to the field conditions. Further studies are needed to clarify the extent of viral transmission by feathers contaminated with AI virus (H5N1).

In conclusion, we report that the feathers of domestic ducks infected with AI virus (H5N1) can be a source of environmental contamination. The possible epidemiological consequence is that contaminated feathers may manifest as fomites containing high viral loads in the environment. People should also be aware that domestic duck feathers are a possible source of zoonotic AI virus (H5N1) infection.

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